mycelium in the presence of phenylacetate resulted in a greater decrease in mycelial phenylacetyl chloride-reacting substances than was found in the absence of phenylacetate.

5. No quantitative correlation was found, however, between the increased penicillin production resulting from the addition of phenylacetate and the decrease in phenylacetyl chloride-reacting substances.

6. The metabolic inter-relationship between 6-aminopenicillanic acid and penicillin is discussed.

We wish to thank Mr E. J. Toms for technical assistance.

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The Ultraviolet Fluorescence of Proteins in Neutral Solution

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The fluorescence characteristic of aqueous solutions of tyrosine, tryptophan and phenylalanine was described in detail by Teale & Weber (1957). The fluorescence of these aromatic amino acids when combined in the polypeptide chains of globular proteins is now reported and some structural implications of the results are discussed.

EXPERIMENTAL

Methods

Fluorescence spectra and fluorescence-excitation spectra. These were determined by means of the apparatus described by Teale & Weber (1957). Protein solutions contained in a quartz cell were irradiated by radiation of 2537 Å wavelength isolated from a low-pressure mercury arc by a combination of a Chance OX7 glass filter and *p*-nitrophenol solution in a silica cuvette. The fluorescence emitted at right angles to the direction of excitation was analysed by a Bausch and Lomb grating monochromator and detected by an E.M.I. 6255B quartz-window photomultiplier. The corrections for varying monochromator transmission and detector response with wavelength are described elsewhere (Teale & Weber, 1957). In certain cases a band of continuous radiation derived from a xenon-arc lamp by means of an aqueous solution of nickel sulphate and *p*-nitrophenol was used to excite over the whole absorption band of the protein.

Fluorescence-excitation spectra were obtained with a hydrogen- or xenon-arc lamp as the continuous source. A filter of Perspex 4 mm. thick or a Chance OX1 glass filter was used to separate exciting light from fluorescence. Cuvettes of extremely pure fused quartz (Thermal Syndicate Ltd., Wallsend, Northumberland, England) were used to reduce cell fluorescence over the 240 mμ excitation range. In the determination of both excitation and fluorescence spectra, experimental points were obtained at wavelength intervals of 5 mμ. Additional points were interpolated where the readings changed rapidly with wavelength.

Fluorescence quantum yields. These were measured by comparing the total emission from protein solutions with that of pure tyrosine or tryptophan solutions with the same excitation absorption. Absolute yields were then calculated by correction for differences in detector response to the fluorescence spectra, assuming the published values for the absolute quantum yields of tyrosine and tryptophan (Weber & Teale, 1957). For accurate measurements of the quantum yield in the wavelength region from 290 to 310 mμ, where the molecular extinction changes rapidly, monochromatic lines of the high-pressure mercury arc were employed to excite fluorescence.

Absorption spectra. These were determined with a Uvispek spectrophotometer at wavelength intervals of 5 mμ, or with an Optika recording spectrophotometer. The extinction coefficients of proteins at wavelengths where the absorption is small were obtained with concentrated

solutions and a dilution factor was applied. The light-scattering by protein solutions, indicated by finite absorption at wavelengths greater than 310 mμ, was reduced to a minimum by filtration or centrifuging.

Propane-1:2-diol–water mixtures of measured viscosity were used to determine the influence of viscosity on fluorescence, and solutions with a wide range of dielectric constants were obtained by using dioxan–water mixtures. Chymotrypsinogen was converted into the active enzyme by trypsin at pH 7·5 (molecular ratio 100:1).

Materials

Pure crystalline proteins for which there were reliable values of aromatic amino acid composition were selected for detailed investigation. Human-serum albumin, crystallized as the mercury dimer by the author, was a gift from Dr J. L. Oncley. Ovalbumin was prepared by the ammonium sulphate fractionation of egg albumin (Warner, 1954). Trypsin inhibitors and trypsin inactivated with di-*iso*propyl phosphorofluoridate were gifts from Dr N. M. Green. Fumarase was a gift from Dr V. Massey. The remaining proteins were available commercially as follows: bovine-serum albumin, haemoglobin, fibrinogen, γ-globulin, lysozyme and pepsin were supplied by Armour Laboratories; trypsin, chymotrypsinogen, chymotrypsin, pepsin, carboxypeptidase and ribonuclease were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; trypsinogen and ribonuclease were products of Sigma Chemical Co., St Louis 13, Mo., U.S.A.; insulin was supplied by British Drug Houses Ltd., as was propane-1:2-diol having high transparency in the ultraviolet region.

Two attempts were made to remove tryptophan selectively from human-serum albumin. In one experiment the protein was irradiated in the presence of methylene blue with a low-pressure mercury arc in the hope of selectively photo-oxidizing tryptophan (Weil & Buchert, 1951). In another experiment the albumin was treated with chymotrypsin and carboxypeptidase simultaneously. As the molecule of albumin contains 17 tyrosine residues but only one tryptophan group, it was hoped that some partly degraded albumin molecules containing only tyrosine would be obtained.

RESULTS

Fluorescence characteristics

Table 1 contains the absolute quantum yields, calculated for equal total absorption at 280 mμ wavelengths, and the fluorescence-band maxima of 21 proteins, each in three different solvents. Table 2 lists the residues of tyrosine, tryptophan and phenylalanine occurring in each molecule of the proteins investigated, and also the fractional absorption of tryptophan at 280 mμ wavelength, calculated from the published molecular-extinction coefficients of tyrosine and tryptophan (Beaven & Holiday, 1952).

Fig. 1 shows typical excitation and fluorescence spectra of tryptophan-containing proteins and also of insulin. The excitation spectrum of human-serum albumin between 220 and 247 mμ is plotted on a changed ordinate scale. All the fluorescence-

Table 1. *Fluorescence yields* (q) *and wavelengths of the maxima of emission for proteins in water, 8 M-urea and propane-1:2-diol*

The overall quantum yields at 280 mμ are denoted by q. The yields denoted by q_1 are calculated assuming that the fluorescence is due to the tryptophan residues alone and that there is no transfer of energy from tyrosine to tryptophan. q_1 = q divided by the fraction of the total absorption due to tryptophan (see Table 2).

	Water			8 M-Urea			Propane-1:2-diol		
Protein	$\lambda_{max.}$ (mμ)	q (%)	q_1 (%)	$\lambda_{max.}$ (mμ)	q (%)	q_1 (%)	$\lambda_{max.}$ (mμ)	q (%)	q_1 (%)
Insulin	304	3·7	—	304	3·0	—	304	8·3	—
Zein	304	8·0*	—	—	—	—	304	8·0	—
Ribonuclease	304	1·7	—	304	1·7	—	304	3·2	—
Ovomucoid	304	1·2	—	304	1·2	—	304	2·0	—
Pancreatic trypsin inhibitor	—	0·0	—	—	0·0	—	—	0·0	—
Bovine γ-globulin	332	3·8	5·0	350	5·3	6·8	337	9·7	12·8
Lysozyme	341	6·0	6·5	350	4·1	4·4	343	6·0	6·45
Trypsin	332	8·1	12·6	350	13·8	21·4	335	14·1	21·8
Trypsinogen	332	8·7	13·4	350	14·0	22·0	335	14·0	21·6
Chymotrypsin	334	9·5	10·5	350	20·4	22·6	338	20·0	22·3
Chymotrypsinogen	331	7·2	8·0	350	20·0	22·2	336	15·8	17·6
Human-serum albumin	339	7·4	38·0	350	5·2	26·2	340	7·4	38·0
Bovine-serum albumin	342	15·2	47·5	350	7·4	23·0	343	15·2	47·5
Ovalbumin	332	12·1	20·9	340	13·1	22·4	336	18·1	31·0
Fumarase	335	9·0	—	—	—	—	—	—	—
Carboxypeptidase†	340	12·2	22·1	348	9·0	16·4	340	14·0	25·3
Pepsin	342	12·8	25·0	350	10·2	20·0	344	18·0	35·3
Fibrinogen	337	14·0	20·6	350	14·0	20·6	340	21·0	31·0
Edestin‡	328	11·8	23·0	350	12·0	23·5	—	—	—
Haemoglobin globin	335	10·0	14·3	348	8·0	11·4	—	—	—

* Ethanol–water (50:50). † 10% Lithium acetate solution. ‡ M-NaCl solution.

Table 2. *Distribution of the aromatic amino acids in proteins and the percentage absorption by tryptophan at 280 mμ wavelength*

Protein	Concn. (mM)	Phenylalanine residues	Tyrosine residues	Tryptophan Residues	Tryptophan Percentage absorption
Lysozyme	15	3	3	8	93
Myoglobin	16·5	4	2	2	80
Trypsin/trypsinogen	24	4	9	4	64·5
Chymotrypsin/chymotrypsinogen	25·1	6	4	7	90
Carboxypeptidase	34·4	15	20	6	55
Pepsin	35	13	16	4	51
Ovalbumin	43	21	9	3	58
Edestin	50	16	12	3	51
Human-serum albumin	65	33	17	1	19·5
Bovine-serum albumin	65	24	18	2	32
Haemoglobin	66	30	11	6	70
Bovine globulin	156	28	18	14	76
Fibrinogen	450	28	30	16	68
Pancreatic trypsin inhibitor	7	5	5	0	0
Insulin	12	6	8	0	0
Ribonuclease	15	3	6	0	0
Ovomucoid	28	5	5	0	0
Zein	50	44	29	0	0

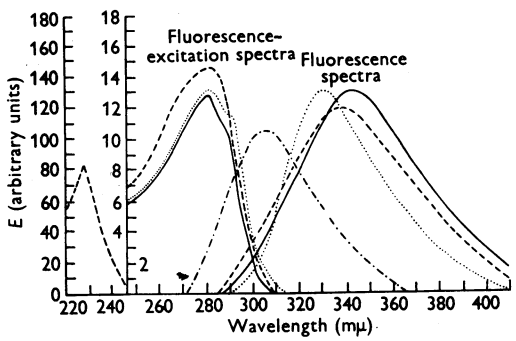

Fig. 1. Fluorescence-excitation spectra and fluorescence spectra of proteins in aqueous solutions. Chymotrypsin (···); insulin (—·—); human-serum albumin (– – –); pepsin (——). In the excitation spectrum of human-serum albumin between 220 mμ and 247 mμ, the ordinate scale of extinction is increased tenfold.

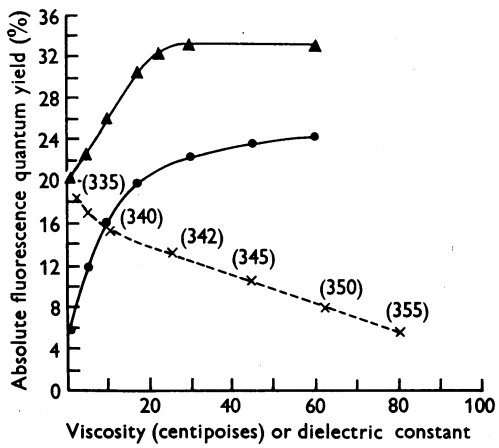

Fig. 2. Influence of the viscosity and dielectric constant of the medium on the fluorescence yields of tryptophan and glycyltryptophan in solution. ▲, Tryptophan yield against viscosity; ●, glycyltryptophan yield against viscosity; ×, glycyltryptophan yield against dielectric constant, with the wavelength (mμ; figures in parentheses) of the fluorescence maximum at selected points.

excitation spectra of proteins containing tryptophan were similar in profile and band maxima, and differed only at the edge of the long-wave-absorption band, where individual proteins showed variations in position accompanied by displacement of the fluorescence spectrum in the converse direction. These spectral shifts were similar to those shown by tryptophan and its derivatives in media of different polarizability, illustrated by Fig. 2, which shows the variation in absolute yield and fluorescence-band maximum of glycyltryptophan with the dielectric constant of the dioxan–water mixtures used as the solvent. Included in Fig. 2 are the variations of absolute yield of tryptophan and glycyltryptophan with solvent viscosity, with propane-1:2-diol–water mixtures.

The proteins listed in Table 1 did not show a fluorescence spectrum with a maximum at 280 mμ, as is given by phenylalanine. The table includes proteins such as human-serum albumin, in which the relative absorption by phenylalanine is large. Pure gelatin and the polypeptide polymyxin, in which phenylalanine is the only aromatic residue, exhibit a fluorescence similar to that of phenylalanine but with reduced quantum yield and showing vibrational structure. Phenylalanine itself shows vibrational structure in the absorption

spectrum but not in the fluorescence spectrum of aqueous solutions. The differences probably reflect the lower polarizability of the molecular environment of combined phenylalanine in gelatin and polymyxin, compared with that of the free amino acid in aqueous solution (Teale & Weber, 1957; F. W. J. Teale, unpublished results).

Proteins containing tyrosine but no tryptophan showed tyrosine fluorescence in low yield or were non-fluorescent. The fluorescence-excitation spectra of insulin and ribonuclease were similar in band position ($\lambda_{max.}$ 227 and 276 mμ) and profile to that of pure tyrosine in water ($\lambda_{max.}$ 224 and 275 mμ) with a slight displacement to longer wavelengths. In the 260 mμ region a contribution of phenylalanine excitation to tyrosine fluorescence was detectable in both proteins.

Proteins containing tryptophan displayed the fluorescence spectrum which is characteristic of this aromatic residue to the exclusion of the tyrosine type of spectrum, even where tyrosine was responsible for most of the absorption at the exciting wavelength. From Table 2, human-serum albumin has relatively the least tryptophan absorption at 280 mμ wavelength. Inspection of the fluorescence spectrum of this protein excited at this wavelength, shown in Fig. 1, reveals a negligible tyrosine contribution at 304 mμ. Comparison of this fluorescence spectrum with that of tryptophan suggests that the quantum yield of the tyrosine fluorescence shown by human-serum albumin cannot be greater than 1·5 %.

Protein–tryptophan fluorescence spectra consisted in all cases of a single band, without the structure sometimes discernible in the absorption spectrum. The excitation spectra were all similar in profile to that of pure tryptophan, with a shift of the band maxima to longer wavelengths ($\lambda_{max.}$ 228 mμ, 282 mμ) compared with tryptophan in water ($\lambda_{max.}$ 218 mμ, 280 mμ).

Fig. 3 (*a*) shows the profiles of the edge of the absorption band of least frequency attributable to tyrosine and tryptophan in proteins. These are obtained from the spectra of proteins in which these aromatic residues predominate and represent average values. Also shown is the variation with wavelength of the fractional absorption of tryptophan in a mixture of tyrosine and tryptophan having equal absorption at 280 mμ wavelength. Fig. 3 (*b*) shows the variation with wavelength of

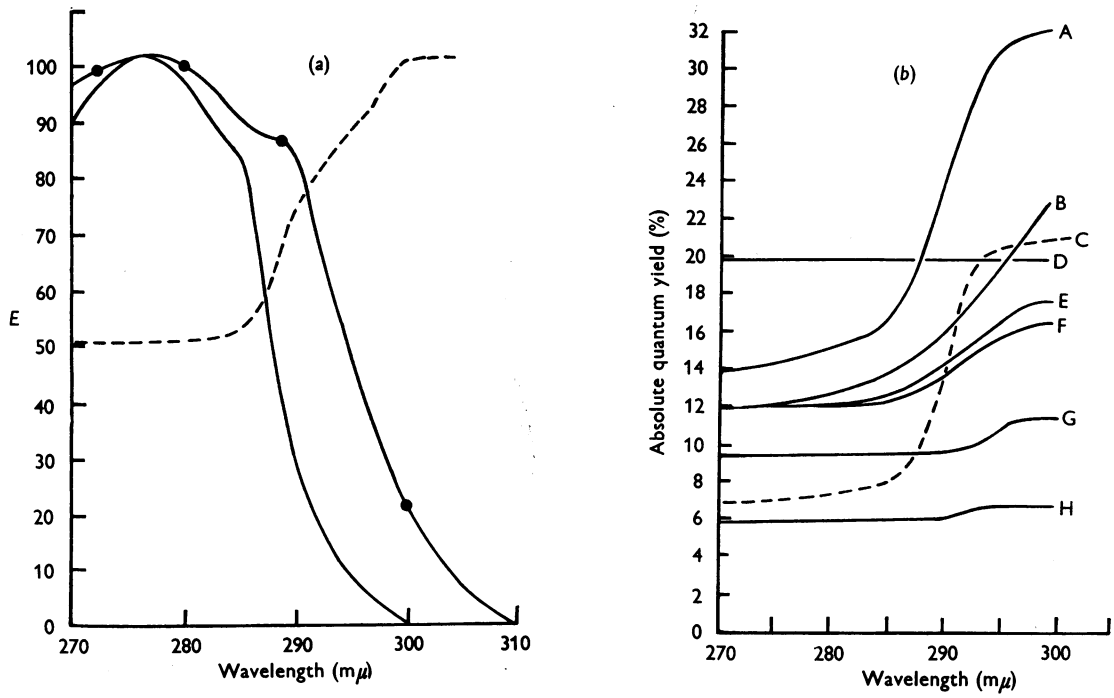

Fig. 3 (*a*). Absorption profiles of tyrosine and tryptophan in proteins between 270 mμ and 310 mμ wavelength. Tyrosine (—); tryptophan (●—●). The fractional absorption by tryptophan in a mixture having equal absorption by both tyrosine and tryptophan at 280 mμ wavelength is shown by the broken line. (*b*) Variation of the fluorescence yield with wavelength of the exciting light in tryptophan and proteins containing tryptophan. A, Bovine-serum albumin; B, pepsin; C, human-serum albumin; D, tryptophan; E, ovalbumin; F, carboxypeptidase; G, chymotrypsin; H, lysozyme.

the absolute fluorescence yield of several tryptophan-containing proteins and of tryptophan in water. These results are considered in more detail in the Discussion section.

Effects of structural changes

Structural changes in proteins produced changes in fluorescence yield and band position. As shown in Table 1, 8M-urea shifted the maxima of all fluorescence spectra of tryptophan-containing proteins to a wavelength of 350 mμ. In some cases it also produced large decreases or increases in fluorescence yield. These changes were sometimes reversed when the urea was removed by dialysis, e.g. chymotrypsin, but the changes were irreversible in other proteins, such as the serum albumins. The specific structural modification involved in the conversion of chymotrypsinogen into the active enzyme was manifested by an increased fluorescence yield and a shifted fluorescence spectrum. 8M-Urea converted both zymogen and enzyme into the same fluorescence parameters, though very slowly with chymotrypsinogen. 0·1M-Calcium chloride reduced the rate of urea-denaturation of chymotrypsin, as measured by the shift of fluorescence spectrum, by a factor of at least 100 (Chervenka, 1959). The trypsinogen–trypsin conversion did not produce large changes in fluorescence, nor did di*iso*propyl phosphorofluoridate-trypsin differ from trypsin.

Increasing the viscosity of the medium by dissolving proteins in propane-1:2-diol almost invariably increased the fluorescence yield, although some proteins were unaffected. Photo-oxidation of the tryptophan content of human-serum albumin in the presence of methylene blue caused the progressive disappearance of all fluorescence; no tyrosine fluorescence was discernible. Treatment of this protein with carboxypeptidase and chymotrypsin produced free tyrosine and tryptophan, which were removed by dialysis. The non-dialysable fraction showed no appreciable tyrosine fluorescence. If the selective removal of tryptophan from albumin was achieved in these experiments, the failure of tyrosine fluorescence to appear indicates a low intrinsic yield for this group in serum albumin.

Quenching of tyrosine

Possible interactions between tyrosine and some of the groups commonly found in proteins were investigated by measuring the fluorescence yield of tyrosine in concentrated aqueous solutions of certain compounds.

Tyrosine was strongly quenched by glycine, glycylglycine, tetraglycine, sodium formate and methyl formate in aqueous solutions and the effects of concentration conformed to the Stern–Volmer equation. The relative concentrations at which the tyrosine yield is reduced to one-half are listed in Table 3. Formamide, urea and ethylamine hydrochloride were ineffective. These results show that strong quenching is produced by the carboxylate group, whereas the charged amino group, amido group and the urea amino groups are without effect. The quenching efficiencies of glycine and related compounds increase with the separation of the charged groups. Surprisingly, the uncharged carboxymethyl group appears almost as effective as the carboxylate ion, although the carbonyl groups in formamide and urea are without effect.

Table 3. *Substances quenching the fluorescence of tyrosine in aqueous solution*

$[q]_{\frac{1}{2}}$ represents that concentration of material at which the fluorescence of μM-tyrosine is halved.

Material	$[q]_{\frac{1}{2}}$ (M)
Tetraglycine	0·110
Sodium formate	0·125
Methyl formate	0·155
Glycylglycine	0·310
Glycine	0·730

The quenching effect of sodium formate was measured with two proteins containing tyrosine. Insulin fluorescence was reduced to half by 0·9M-sodium formate, whereas that of human-serum albumin was unaltered by a 2·0M solution.

DISCUSSION

In the protein molecule each aromatic group can interact in the ground or excited states with adjacent chemical groups of the polypeptide structure, which may be relatively inert paraffin side chains or phenyl groups, or reactive hydrophyllic and charged groups. The displacement of the absorption spectra of tyrosine and tryptophan in proteins compared with aqueous solutions (Beaven & Holiday, 1952) and absorption attributable to tyrosine-carboxylate hydrogen bonds (Tanford, Hauenstein & Rands, 1955) both show that ground-state interactions between these aromatic amino acids and the protein environment occur, which together with possible interactions in the singlet excited state could be expected to reduce the quantum yields shown by these residues in proteins (Teale & Weber, 1959).

The possibilities of energy transfer must also be considered. The relative positions of the absorption and fluorescence spectra should, in favourable circumstances, permit resonance-energy transfer from phenylalanine and tyrosine to tryptophan, and also between adjacent tyrosine groups. Recent fluorescence-polarization measurements with insulin, ribonuclease and zein show clearly that

intertyrosine transfer does indeed occur in these proteins (G. Weber, unpublished work). All the aromatic residues are brought into close proximity by their incorporation into protein molecules, and the average distance between residues is in the range at which efficient transfer is known to take place in other systems (Weber, 1954). For a regular array of aromatic residues, the average spacing, $\bar{D}$ (Å) is given by the expression:

$$\bar{D} = \sqrt[3]{\frac{M}{0{\cdot}602nd}},$$

where M is molecular weight, d is protein density and n is number of aromatic residues in the protein molecule. Values of $\bar{D}$ for the tyrosine groups in the proteins investigated are listed in Table 4. The regular array of groups in the protein to which $\bar{D}$ corresponds is the arrangement least favourable for transfer, so that the more random distribution which undoubtedly exists in proteins should favour the predicted energy transfers. Efficient intertyrosine transfer would permit energy migration throughout those proteins in which this residue predominates. It might be expected that tryptophan fluorescence would be excited by light absorbed by phenylalanine and tyrosine. In fact, phenylalanine fluorescence was not detected in the presence of other aromatic residues, and those proteins containing tryptophan showed the fluorescence of this residue exclusively, irrespective of the relative tyrosine content. The virtual absence of tyrosine fluorescence does not in itself establish complete energy transfer to tryptophan, however; the alternative possibility that tyrosine in proteins may be quenched was suggested by the low fluorescence yields of two trypsin inhibitors and ribonuclease. The investigation of these alternatives was made difficult by the similarity of the absorption spectra of tyrosine and tryptophan. These spectra differ in profile at the absorption minima (approx. 250 mμ) and also at the absorption edge in the wavelength range 280–310 mμ. Measurements at the absorption minimum in proteins were invalidated by the possible transfer from phenylalanine and also by uncertainties of the tyrosine-absorption spectrum in this region.

Table 4. *Average distance* ($\bar{D}$) *between the tyrosine groups in proteins*

Protein	$\bar{D}$ (Å)
Pancreatic trypsin inhibitor	14·73
Zein	15·74
Carboxypeptidase	15·74
Insulin	16·57
Ribonuclease	17·0
Pepsin	17·1
Trypsinogen	18·22
Trypsin	18·22
Human-serum albumin	19·31
Bovine-serum albumin	20·17
Edestin	21·14
Ovalbumin	22·17
Lysozyme	22·51
Ovomucoid	23·33
Chymotrypsinogen	23·33
Chymotrypsin	23·33
Haemoglobin	23·87
Myoglobin	26·54
Bovine γ-globulin	27·05
Fibrinogen	32·30

Less equivocal evidence was furnished by a detailed examination of the variation of tryptophan fluorescence yield over the excitation range 280–310 mμ, where it can be assumed that almost all the absorption is due to tyrosine and tryptophan. In this region the two residues differ in absorption profile, tryptophan absorption extending towards longer wavelengths compared with tyrosine, so that in a mixture of these residues changes in fractional absorption occur, shown in Fig. 3 (*a*). Uniform quantum yields would be observed in this region if efficient energy transfer took place from tyrosine to tryptophan. Experimental results shown in Fig. 3 (*b*) indicate that this is not the case. The large changes in yield observed mean that tyrosine excitation does not contribute appreciably to tryptophan fluorescence. The increases in yield shown in Fig. 3 (*b*) must be regarded as minimal values since it is by no means certain that tryptophan is responsible for all the absorption in the region 295–300 mμ. This is certainly not so when cystine is present in large amounts, as in the serum albumins. By comparison of these yield increases with the fractional absorptions included in Table 2, it is possible to set an upper limit to the amount of transfer present in the proteins investigated. This amount is negligible in all cases except the serum albumins, where transfer to tryptophan from one tyrosine residue at most is indicated. The results of this investigation are in agreement with the two attempts to remove tryptophan preferentially from human-serum albumin. Photo-oxidation progressively reduced the absorption at wavelength 280 mμ and the fluorescence band at 339 mμ without revealing tyrosine fluorescence ($\lambda_{max.}$ 304 mμ). Similarly, the enzymic degradation rapidly liberated dialysable material containing tryptophan, but at no stage was tyrosine fluorescence detectable in the remaining non-dialysable fraction. Both these results suggested that tyrosine residues in albumin are virtually non-fluorescent.

The great similarity between the fluorescence excitation spectra of all these proteins and tryptophan itself rules out any large contribution to protein fluorescence by non-aromatic absorption attributable to cystine, histidine or to the peptide linkage itself, as has been suggested (Konev, 1959).

In Table 1, tryptophan-fluorescence yields, calculated on the assumption of no energy transfer by

dividing the overall yields listed by the tryptophan fractional absorptions listed in Table 2, are included to the right of the overall values in the different media. The wide range of tryptophan-fluorescence yields found in proteins reflects the relative efficiency of quenching processes. For most proteins the yields are reversibly raised by dissolving the protein in propane-1:2-diol, which completes for hydrogen bonds and reduces the rotational freedom of groups on the protein surface. The two serum albumins are unaffected by this treatment, either because the tryptophan groups are in a hydrophobic environment not accessible to the glycol, or because no quenching occurs. The latter possibility is suggested by the high intrinsic yield. In lysozyme, which has a low yield unaltered by glycol, it is unlikely that all eight tryptophan groups cannot be in contact with the medium.

The effect of 8M-urea is more complicated, but generally proteins unaffected by glycol are quenched by 8M-urea, whereas proteins whose yield is increased by glycol are increased by urea, so that the intrinsic tryptophan yields (q_1) become more nearly uniform for all the proteins investigated. With the exception of γ-globulin, lysozyme, haemoglobin and carboxypeptidase, the tryptophan yield in 8M-urea was in all cases $23 \pm 3\%$, and the fluorescence spectra all showed the same band maximum at 350 mμ.

These changes are understandable if urea disrupts the unique protein configuration which provides the specific quenching environment of each tryptophan residue and thus produces a more uniform environment for all the tryptophan groups, and hence a more uniform yield. This effect is clearly seen in the urea denaturation of chymotrypsinogen and chymotrypsin. The fluorescence yields and spectra are originally different but become almost identical after treatment with urea. The fluorescence changes which accompany the activation of chymotrypsinogen are due to a decrease in the quenching of one or more of the tryptophan groups. Presumably, the same structural changes are responsible for the changes in the absorption which are attributable to tyrosine (Chervenka, 1959).

The mechanism responsible for the quenching of tyrosine fluorescence in proteins is suggested by a comparison of insulin with ribonuclease. It is well established that half of the tyrosine phenolic groups in ribonuclease are involved in hydrogen bonds to carboxylate groups, whereas the same groups in insulin appear to be free (Tanford & Epstein, 1954, Tanford *et al.* 1955). The low yield given by ribonuclease (1·7%) compared with insulin (3·7%) is explained if tyrosine is quenched by the interaction with carboxylate. That this is indeed the case is shown by the quenching action of the formate anion and, to a lesser extent, glycine and glycine peptides. Other groups found in proteins, the amide and charged amino groups, seem to be ineffective. The tyrosine-carboxylate hydrogen bond increases the pK of the ionization of the phenolic group (Laskowski & Scheraga, 1954), and the pK of this group in proteins, measured spectrophotometrically, is a measure of the phenolic hydrogen bond. A correlation exists between tyrosine fluorescence and the pK values found in proteins. Insulin shows appreciable fluorescence and a tyrosine pK of 9·7, which is similar to that of free tyrosine. Ribonuclease with roughly half the yield of fluorescence of insulin has half of its tyrosine groups with pK 10·2 and the remainder with pK greater than 11·5. Ovalbumin, in which all the tyrosine groups have a pK greater than 11·5, shows no tyrosine fluorescence. The two serum albumins also show high pK values (Tanford & Roberts, 1952). It must be kept in mind that absence of tyrosine fluorescence does not imply the involvement of all the phenolic groups because energy transfer to quenched tyrosine can decrease the yield of those groups still capable of fluorescence, but measurements indicate that in most of the proteins investigated a large fraction of the phenolic groups is involved in quenching interactions, probably hydrogen bonds to neighbouring carboxylate groups.

Many observations support the view that these phenolic linkages share with cystine the maintenance of the protein tertiary structure. The phenolic dissociation is reversible in the serum albumins and lysozyme, which have relatively high cystine contents (Tanford & Wagner, 1954), but is irreversible in ovalbumin, which has only one cystine bridge (Crammer & Neuberger, 1943) and which is also irreversibly denatured when all the carboxylate groups are titrated at pH 2·0 (Gibbs, Bier & Nord, 1952). Protein-denaturation is frequently accompanied by the freeing of phenolic groups bound in the native protein. For example, heat-denaturation of ribonuclease equalizes the pK values of all six tyrosine groups to the lower value (pH 10·2) shown by only three tyrosine groups in the native protein (Tramer & Shugar, 1959). This agrees with the observation that the fluorescence yield of ribonuclease is doubled by heat-denaturation.

SUMMARY

1. The fluorescence spectra, fluorescence-excitation spectra and absolute quantum yields of 21 globular proteins have been measured in neutral solutions.

2. Phenylalanine fluorescence was not observed in the presence of other aromatic groups, and

tyrosine fluorescence was detected in low yield with band maximum at 304 mμ only in the absence of tryptophan.

3. In proteins containing tryptophan the fluorescence spectrum of this residue alone is observed, irrespective of the wavelength of the exciting light, even in those cases where tyrosine predominates in absorption. The band maxima vary in wavelength between 328 and 342 mμ, and the overall quantum yields 3·8–15·2% in water.

4. Detailed examination of the change in tryptophan quantum yield in the excitation region between 280 and 310 mμ reveals that energy transfer from tyrosine to tryptophan does not take place to any appreciable extent. The similarity of all protein-excitation spectra with that of either tyrosine or tryptophan shows that light absorbed by cystine or the peptide bond does not contribute to protein fluorescence.

5. Modification of the protein structure by 8M-urea produces large and in some cases reversible changes in tryptophan fluorescence, leading to similar yields and fluorescence spectra in most of the proteins examined. The slight structural change involved in the activation of chymotrypsinogen increases the quantum yield by 32%. Propane-1:2-diol increased the yield of all the proteins except the serum albumins and lysozyme.

6. The absence of tyrosine fluorescence in many proteins is explained by the quenching action of the tyrosyl-carboxylate hydrogen bond, and is correlated with the pK values for the phenolic ionization. The role of this linkage in the maintenance of the protein tertiary structure is briefly discussed.

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The Biochemistry of Aromatic Amines

7. THE ENZYMIC HYDROLYSIS OF AMINONAPHTHYL GLUCOSIDURONIC ACIDS*

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As 2-naphthylamine induces cancer of the bladder only, in men exposed to the amine, the actual carcinogen is probably a metabolite of the amine (Boyland, 1958). When tested by implantation in the mouse bladder 2-naphthylamine was much less carcinogenic than one of its metabolites, 2-amino-1-naphthol (Bonser, Clayson, Jull & Pyrah, 1952; Bonser, Clayson & Jull, 1958). However, the 2-amino-1-naphthol has been detected in fresh urine only in conjugated forms (Boyland, 1958) and Boyland (1956) has suggested that it is necessary for the conjugates to be hydrolysed before carcinogenesis occurs. 2-Amino-1-naphthyl sulphate is not hydrolysed by urinary sulphatase (Boyland, Manson, Sims & Williams, 1956) and is not carcinogenic in the mouse bladder (Bonser, Bradshaw, Clayson & Jull, 1956). The corresponding glucosiduronic acid, however, can be hydrolysed by mammalian β-glucuronidase preparations (Boyland & Manson, 1957) and is carcinogenic in the

* Part 6: Boyland & Williams (1959).